Traffic Related Air Pollution, Oxidative Stress Genes and Asthma (ECHRS)

Francesc Castro-Giner, Nino Künzli, Bénédicte Jacquemin, Bertil Forsberg, Rafael de Cid, Jordi Sunyer, Deborah Jarvis, David Briggs, Danielle Vienneau, Dan Norback, Juan R González, Stefano Guerra, Christer Janson, Josep-Maria Antó, Matthias Wjst, Joachim Heinrich, Xavier Estivill, Manolis Kogevinas.

Online Data Supplement

Material and methods

Study population

The ECRHS is a population-based multicentric cohort study. Subjects mostly from countries members of the EU at that time, were randomly recruited in the early 1990s (ECRHS I) and then followed approximately 10 years later. ECRHS I consisted of two phases, in a first step a random sample of the population aged 20-44 years living in the study areas was contacted and asked to complete a short questionnaire on respiratory symptoms.(Burney et al. 1994)

In the second step, an approximately 20% random sub-sample of stage 1 participants was re-contacted to complete a long questionnaire and undergo some exams. A complementary sample including all symptomatic subjects from stage 1 who had not been selected in the random sample was also invited for health assessment. These subjects reported at least one of the following characteristics: an attack of asthma in the last 12 months, be woken by an attack of shortness of breath in last 12 months or be currently taking any medicines for asthma. (European Community Respiratory Health Survey II Steering Committee 2002) Subjects in most centres were followed up with a median length of follow-up time of 8.9 years from the first phase (ECRHS I) to the second phase (ECRHS II). For ECRHS II, modelled NO_2 concentrations were assigned to a total of 5470 participants in the 20 centres for which modelled air pollution was available (see below). From those, DNA was collected in 13 centres with a total of 2920 participants. For this analysis we excluded subjects from the symptomatic sample that did not report current asthma in ECRHS II, leading to a final sample size of 2577.

Subjects included in our analysis could be considered as mainly of European-Caucasian origin. Ethical approval was obtained for each centre from the appropriate institutional ethics committee and written consent was obtained from each participant. The presence of asthma was based on a positive response to either of two questions: attack of asthma during the 12 months preceding the interview or current use of asthma medication. Among subjects reporting an asthma attack, 72% also reported use of asthma medication. New-onset of asthma was defined as reporting asthma (either attack of asthma in last 12 month or current medication for asthma) in ECRHS II evaluation but not in ECRHS I, and persistent asthma was defined as reporting asthma (either attack of asthma in last 12 month or current medication for asthma) in both stages.

We evaluated the robustness of our results with other definitions of asthma. Ever asthma was defined as positive response to “have you ever had asthma” and physician diagnosed asthma as positive response to “have you ever had asthma diagnosed by a doctor”. Participants also underwent a bronchial challenge with methacholine chloride administered by MEFAR® aerosol dosimeters (Mefar, Bovezzo, Italy). BHR was defined as a 20% fall in FEV1 from the highest FEV1 post-diluent during methacholine challenge with an accumulated dose of 1 mg. Additional details on the challenge have been described elsewhere (Burney et al. 1994, Chinn et al. 1997). Specific IgE levels to house dust mite (*Dermatophagoides pteronyssinus*), cat, timothy grass and *Cladosporium herbarum* were measured with the Pharmacia CAP system (Pharmacia Diagnostics, Uppsala, Sweden). Atopy was defined as sensitization (IgE levels >0.35 kU/L) to any allergen.

Modelled NO_2 concentrations

NO_2 has been widely used as a marker of local traffic-related air pollution.(Emenius et al. 2003, Forastiere et al. 2006, Jacquemin et al. 2008, Modig et al. 2006, Morgenstern et al. 2008) The value of NO_2 measurement substantial contrasts within cities as it captures differences in exposure due to different proximity to traffic arteries. Details on modelling of NO_2 concentrations are described elsewhere.(Jacquemin et al. 2008) Briefly, as part of the Air Pollution Modelling for Support to Policy on Health and Environmental Risk in Europe project (APMoSPHERE) project (APMoSPHERE 2007), 1-km-resolution emission maps were developed. The NO_x emission map was used as the basis for modelling NO_2 concentrations using focal sum techniques in a GIS model. The model estimates concentrations by calibrating the distance-weighted sum of the emissions (tonnes/km/year) in concentric circles around each monitoring site to the monitored concentrations ($\mu g/m^3$). The NO_2 at the place of residence for each subject was then obtained by intersecting the point locations of their residence with the air pollution map. This analysis was restricted to 13 centres from 6 European countries with DNA avaliable: Sweden (Umeå and Uppsala), United Kingdom (Ipswich and Norwhich), Spain (Albacete, Barcelona, Huelva, Galdakao and Oviedo), Germany (Erfurt), France (Paris and Grenoble) and Belgium (Antwerp).

Models are developed by setting the weight of the innermost annulus to 1, and each successively outer annuli (to a maximum of 11 km) to $W_{a-1}/2$ (where W_{a-1} is the weight of the next, inner annulus). Weights for each of the annuli were then incrementally adjusted, from the second annulus outwards under the rule that $W_a <= W_{a-1}$, and the correlation with the monitored concentrations recomputed, until R^2 was maximized. The resulting regression model was then used to convert the sum of the weighted emissions to a concentration (in $\mu g/m^3$).

Models were developed using monitoring data from 714 background sites for the year 2001, drawn from the EU Airbase database. Validation was conducted by comparing predictions with observations for a separate set of 228 reserved background sites (r^2 = 0.60). The resulting model was converted into a kernel file (with weights for each annulus), which was then moved across the EU emissions map to produce a 1-km gridded map of concentrations.

Genotyping

The polymorphisms of genes *GSTM1* (deletion), *GSTT1* (deletion), *GSTP1* (Val105Ile: rs16951) were selected only according to functional evidences from existing literature. For the genes *NQO1* (Pro187Ser: rs1800566, rs10517, rs2917666), *TNFA* (rs1800629, rs2844484, rs909253), *TLR4* (rs10759930, rs11536889, rs1554973, rs2737191, rs1927914) and *ADRB2* (rs1042713, rs1042714, rs1042718, rs1042719), we selected tagSNPs in the gene region and 10 kb upstream from 5'UTR and 10 kb downstream from 3'UTR derived from the HapMap Project (http://www.hapmap.org , phase I, release #16) and Perlegen database (http://genome.perlegen.com/). After evaluating SNP coverage for all candidate genes we used preferentially tagSNPs based on r^2 multimarker method, but selecting those SNPs with a known functional effect from the SNPs belonging to a bin.

DNA bank was built and maintained at Helmholtz Zentrum München in Germany. Genotyping was performed at the Centre for Genomic Regulation (CRG) in the Barcelona Node of the "Centro Nacional de Genotipado" (CeGen) in Spain (http://www.cegen.org). *GSTM1* and *GSTT1* genotypes were determined using PCR method and *GSTP1* Ile105Val (rs16951) polymorphisms by specific pyrosequencing assay. Genotyping for *NQO1*, *TNFA* and *TLR4* polymorphisms was performed using the

SNPlex™ platform (Applied Biosystems, Foster City, CA). The average genotyping rate was 98%.

Statistical analysis

The statistical analyses were performed using logistic regression implemented in the SNPassoc (Gonzalez et al. 2007) R package (version 2.6.1).(R Development Core Team 2007) General additive models (GAM) were used to evaluate dose-response relationship with NO_2. Logistic and GAM models were adjusted for centre, sex, age, environmental tobacco smoke and smoking status. Multiplicative interactions were assessed using likelihood-ratio test comparing models with additive term and interaction term. Heterogeneity was evaluated using Mantel-Haenszel method under fixed effects model with R library rmeta version 2.14. Logistic mixed-effects models allowed the evaluation of a random effect of the variable centre. These models were also adjusted for the previously described covariates.

We tested deviations of genotype frequencies from Hardy-Weinberg equilibrium (HWE). (Wigginton et al. 2005) D', R^2 and χ^2 p-values for marker independence were estimated to determine linkage disequilibrium between genetic markers. Haplotypes were estimated using haplo.em function from haplo.stats package (version 1.3.8). (Schaid et al. 2002) Population stratification was assessed with the analysis of 26 unlinked markers (See Supplemental Material, Table 2) using two different methods. First, genomic control (GC) approach (Devlin et al. 1999) performed in an earlier paper (Castro-Giner et al. 2008) showed a minimal effect (inflation factor (λ) = 1.06). Second, principal component analysis using EIGENSTRAT method (version 1.01) (Price et al. 2006) showed no evidence of population stratification (See Supplemental Material, Figure 1).

Supplemental Material, Table 1. Characteristics of the three different sets of ECRHS II population with DNA, with estimated NO_2 exposure and with both NO_2 and DNA.

	Subjects with DNA	Subjects with estimated NO_2 exposure	Subjects with both NO_2 measured and DNA
Subjects, n	5065	5470	2577
Females, n (%)	2643 (52.2)	3053 (55.8)	1345 (52.2)
Age, mean (sd)	42.71 (7.2)	42.97 (7.2)	43.03 (7.3)
Smoking status			
Never Smokers, n (%)	2195 (43.4)	2360 (43.3)	1130 (43.9)
Ex smokers, n (%)	1294 (25.6)	1513 (27.8)	714 (27.8)
Current smokers, n (%)	1568 (31.0)	1578 (28.9)	729 (28.3)
NO_2 measurement ($\mu g/m^3$)			
Percentil 25	-	20.7	20.4
Percentil 50	-	27.8	27.7
Percentil 75	-	33.3	33.5
Have you ever had asthma, n (%)	886 (17.5)	885 (16.2)	432 (16.8)
Asthma symptoms in last year and current medication for asthma, n (%)	553 (11.1)	556 (10.9)	327 (12.7)

Supplemental Material, Table 2. Characteristics of SNPs selected for assessment of population stratification.

SNP	Chromosome	Position	Gene	Major allele frequency	P value HWE	% Genotype rate
rs1490413	1	4277696	-	56.4	0.75	99,4
rs10495407	1	234765349	-	66.1	0.82	99,2
rs876724	2	104974	-	69.1	0.41	98,9
rs4988235	2	136442378	LCT	57.0	<0.001	99,0
rs309125	2	136477287	LCT	65.0	<0.001	99,1
rs907100	2	239345579	-	55.5	0.34	98,6
rs1357617	3	936782	-	71.8	0.48	99,5
rs1979255	4	190693229	-	66.6	0.97	99,4
rs717302	5	2932395	-	50.5	0.24	99,0
rs1029047	6	1080939	-	63.7	0.03	99,5
rs917118	7	4230244	-	71.9	0.46	98,3
rs2056277	8	139468298	-	73.3	1.00	99,7
rs1015250	9	1813774	-	80.0	0.76	99,8
rs2076848	11	134172756	-	57.2	0.82	98,8
rs2111980	12	104830721	-	56.0	0.19	99,6
rs1335873	13	19799724	-	74.5	0.18	99,3
rs873196	14	97915284	-	60.7	0.03	86,5
rs1900758	15	25903692	OCA2	66.4	0.16	91,0
rs1800404	15	25909368	OCA2	77.7	0.01	99,4
rs729172	16	5546198	-	58.7	0.98	99,55
rs740910	17	5647347	-	70.4	0.97	98,1
rs1024116	18	73561374	-	57.2	0.09	86,41
rs719366	19	33155177	-	62.9	0.05	98,94
rs1005533	20	38920524	-	54.0	0.84	99,67
rs722098	21	15607469	-	80.6	0.11	99,37
rs2040411	22	46156931	-	66.0	0.17	98,69

Supplemental Material, Table 3. Distribution of modelled NO_2 (per 10 $\mu g/m^3$) and current asthma by centre.

			NO_2 percentiles				
Centre	n	Median NO_2 (sd)	P25	P50	P75	Cases of Current asthma	Percentage
Antwerp, Belgium	506	27.9(5.5)	23.2	27.8	32.9	29	6.2
Grenoble, France	237	27.5(6.9)	25.4	30.6	31.5	24	10.8
Paris, France	311	47.4(9.8)	49.1	50.5	52.6	21	6.8
Erfurt, Germany	66	24.1(4.7)	22.1	25.4	27.7	4	6.1
Albacete, Spain	133	28.4(3.8)	28.3	29.8	31.8	8	7.8
Barcelona, Spain	190	54.4(9.3)	53.9	57.3	58.8	25	15.7
Galdakao, Spain	331	25.8(7.8)	20.1	24.6	33	25	8.8
Huelva, Spain	184	31.4(4.4)	29.7	33.4	33.7	16	12.3
Oviedo, Spain	115	28.8(5)	26.5	30.5	32.1	10	11.8
Umea, Sweden	93	12.2(2.4)	10.6	12	13.9	23	28
Uppsala, Sweden	437	15.6(5.2)	11.9	15.3	19.3	77	20.4
Ipswich, UK	112	26.2(2.9)	25.1	26.1	27.1	24	24
Norwich, UK	205	24.1(4)	22.5	25.3	26.6	41	21.2

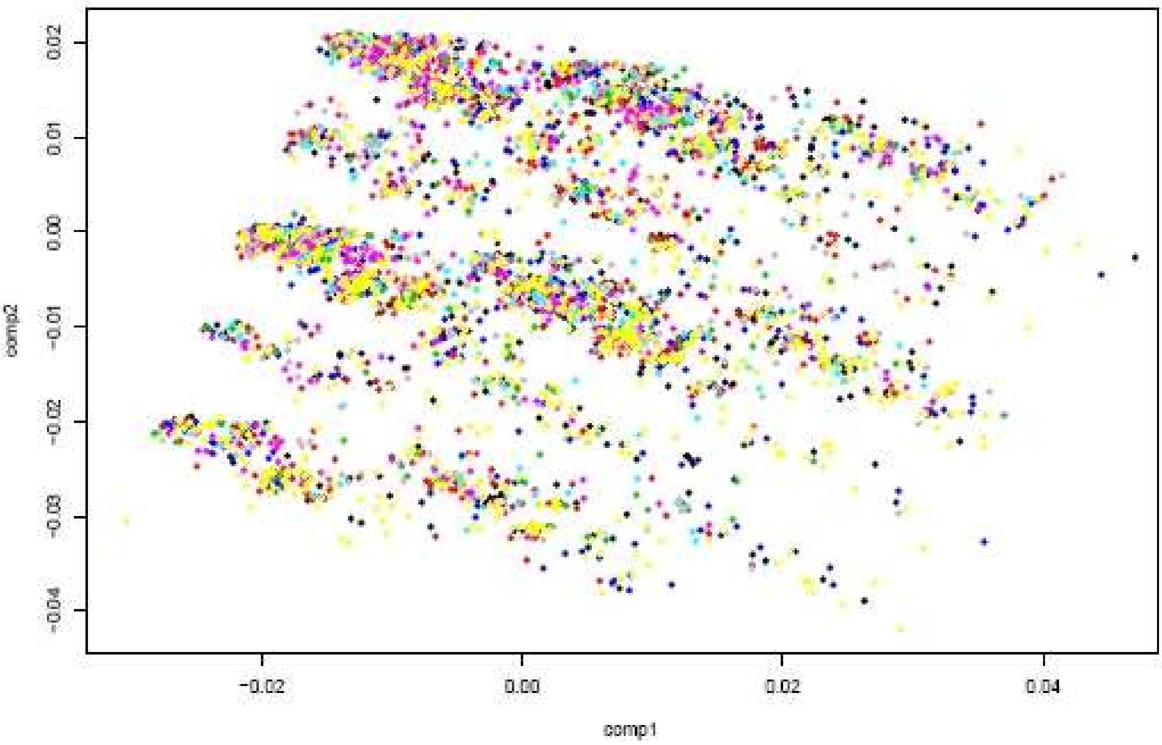
Supplemental Material, Table 4. NO_2 effect on different phenotypes by *NQO1* rs2917666 genotypes. Model adjusted by: centre, sex, smoking status, environmental tobacco smoking status and age

		NQO1 rs2917666 C/C		*NQO1* rs2917666 C/G or G/G		P-value interaction	
Phenotype	n	Case/control	OR (95%CI)	Case/control	OR (95%CI)	Dominant model	Additive model
Current asthma prevalence	327	155/1051	1.54 (1.1-2.24)	171/1197	1.01 (0.79-1.33)	0.04	0.02
Medication in last 12 m	263	125/1226	1.61 (1.1-2.44)	137/1419	1.10 (0.81-1.5)	0.008	0.004
Attack of asthma last 12 m	223	104/1246	1.51 (1.01-2.36)	118/1444	1.14 (0.82-1.61)	0.05	0.008
Ever asthma	503	238/1114	1.39 (1.07-1.83)	264/1300	1.10 (0.89-1.38)	0.003	0.006
Doctor diagnosed asthma	471	217/1134	1.35 (1.03-1.79)	253/1311	1.08 (0.87-1.36)	0.005	0.01
Current asthma and BHR	121	60/597	2.29 (1.27-4.58)	61/722	1.36 (0.88-2.21)	0.03	0.02
New-onset of asthma	120	54/1040	2.02 (1.16-3.73)	65/1181	1.26 (0.83-1.99)	0.03	0.04
Persistent asthma	203	98/1040	1.31 (0.87-2.06)	105/1181	0.90 (0.67-1.25)	0.24	0.12
Atopy	983	450/885	0.99 (0.81-1.21)	485/1054	0.99 (0.84-1.18)	0.35	0.46
Atopic asthma	229	115/750	1.30 (0.88-2.02)	114/876	1.30 (0.88-2.02)	0.50	0.45
Non-atopic asthma	91	38/750	2.41 (1.13-5.78)	52/876	0.91 (0.58-1.49)	0.02	0.01

Supplemental Material, Table 5. NO_2 effect on asthma prevalence by *NQO1* rs2917666 genotypes according to gender. Model adjusted by: centre, sex, smoking status, environmental tobacco smoking status and age

			Males				Females				
Gene	Polymorphism	Genotype	Case/Control	OR (95%CI)	pval	pIadd	Case/Control	OR (95%CI)	pval	pIadd	P Heterogeneity
NQO1	rs2917666	C/C	63/507	1.391 (0.82-2.46)	0.236	-	92/544	1.812 (1.13-3.09)	0.02	-	
NQO1	rs2917666	C/G+G/G	73/588	1.152 (0.78-1.76)	0.494	0.243	98/609	0.908 (0.64-1.32)	0.60	0.03	0.71

Supplemental Material, Figure 1. The figure shows the first two axes of genotypic variance for each of the nine countries, using EIGENSTRAT procedure as described in Price et al, 2006. (Price et al. 2006) EIGENSTRAT, uses principal components analysis to explicitly model ancestry differences between control and cases (current asthma). Each point represents each one of the 5,065 individuals who were genotyped. Colours represent the different countries included in the study. The plot indicates no subdivision within the population.



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